Analysis of the Respiratory Syncytial Virus Fusion Protein Using Monoclonal and Polyclonal Antibodies

By EDWARD E. WALSH,1* PAUL J. COTE, 2 BRUCE F. FERNIE,2 JACOB J. SCHLESINGER1 AND MICHAEL W. BRANDRISST

1Department of Medicine, The Rochester General Hospital and the University of Rochester School of Medicine and Dentistry, Rochester, New York 14621 and 2Division of Molecular Virology and Immunology, Department of Microbiology, Georgetown University Schools of Medicine and Dentistry, Rockville, Maryland 20852, U.S.A.

(Accepted 12 November 1985)

SUMMARY

At least four distinct epitopes are described on the respiratory syncytial virus fusion protein (VP70) using 13 monoclonal antibodies in solid-phase competitive binding studies. Two, and possibly three, fusion-inhibiting epitopes, one non-fusion-inhibiting neutralizing epitope, and one non-neutralizing epitope are described. All but the latter site demonstrated partial overlap, suggesting possible topographical proximity of these epitopes. Polyclonal rabbit sera to VP70, which neutralized virus but did not inhibit fusion of infected cells, blocked the binding of all fusion-inhibiting monoclonal antibodies to VP70 in the solid-phase assay but did not inhibit their effect in vitro. Western blot analysis of these monoclonal antibodies demonstrates that one fusion-inhibiting epitope is localized on the 48K fragment of VP70 and is resistant to denaturation by heat, 2-mercaptoethanol and SDS.

INTRODUCTION

Respiratory syncytial virus (RSV), an enveloped RNA virus of the paramyxovirus family, is the major cause of severe lower respiratory tract infection in infants and young children (Kim et al., 1973). Attempts at vaccination with both killed and live attenuated vaccines have been unsuccessful and a useful RSV vaccine is currently not available (Kim et al., 1969; Belshe et al., 1982). Recent efforts have been directed at characterization of those RSV proteins which induce biologically important antibodies, with the hope that this information will be useful in the development of a successful vaccine.

Similar to other paramyxoviruses, RSV has two envelope glycoproteins which mediate infectivity and spread of virus. A 90000 mol. wt. (90K) glycoprotein, GP90, probably initiates infection via attachment of the virus to the host cell (Walsh et al., 1984a). A 70K glycoprotein, VP70, is the RSV fusion protein (Walsh & Hruska, 1983). The fusion protein, composed of disulphide-linked fragments of 48K and 23K, probably mediates virus entry into cells by fusion of viral and cellular membranes (Fernie & Gerin, 1982). The fusion protein induces syncytium formation in tissue culture by fusing the membranes of infected cells to adjacent uninfected cells, thereby promoting cell–cell spread of virus. Similar to other paramyxoviruses (Merz et al., 1981), antibody to the attachment protein (GP90), although capable of neutralizing virus, is unable to halt cell–cell spread of virus since syncytium formation is not inhibited (Walsh et al., 1984a). Antibody to the fusion protein, on the other hand, neutralizes virus and inhibits syncytium formation, thereby effectively preventing cell–cell spread of virus (Walsh & Hruska, 1983; Walsh et al., 1985). However, monoclonal and monospecific polyclonal antibodies to the fusion protein which neutralize virus but do not inhibit syncytium formation have been described (Fernie et al., 1982; Walsh et al., 1985), thus suggesting that antibody must bind to a specific epitope on the fusion protein in order to inhibit its function.
In this report, we describe the topographical map of the RSV fusion protein including fusion-inhibiting and neutralizing epitopes, utilizing both monoclonal and polyclonal antibodies. The results are discussed with regard to vaccine development and the evaluation of RSV strain differences.

METHODS

Virus and cells. HEp-2 cells were maintained in MEM supplemented with 2 mM-glutamine and 5% foetal calf serum. The Long strain of RSV was used for all experiments and had a titre of \(2 \times 10^6\) p.f.u./ml.

Monoclonal antibodies (MAbs). Thirteen MAbs to VP70 were used. Seven of these (L4, R661, R392, R13-1, R136, 332-4, R294) have been previously described while six others have not (Taylor et al., 1984; Fernie et al., 1982; Walsh & Hruska, 1983). R235 was produced by immunizing a mouse with a BALB/c cell line persistently infected with RSV as described by Fernie et al. (1982). Five additional MAbs were produced by immunizing a BALB/c mouse with 4 \(\mu\)g of purified VP70 in complete Freund’s adjuvant (Walsh et al., 1985). Four weeks later 4 \(\mu\)g of VP70 in incomplete Freund’s adjuvant was given followed by a second boost of 2 \(\mu\)g of VP70 at 6 weeks. The spleen cells were fused with P3-X63-Ag8.653 cells according to published methods (Fazekas de St. Groth & Scheidegger, 1980). After screening by indirect immunofluorescence, secreting hybridomas were subcloned by limiting dilution. Mouse ascitic fluid containing the MAbs was used in all experiments. The total protein content of each ascitic fluid was determined by Coomassie Brilliant Blue assay (Sigma) and electrophoresis on cellulose acetate membranes was performed. The preparations were then scanned and the areas under the curves integrated by densitometry. The MAbs peaks were identified and protein concentrations of these were calculated as a percentage of the total protein. The concentration of MAb ranged from 4-5 to 24-6 mg/ml.

Polyclonal rabbit antisera to VP70. Two previously described monospecific rabbit antisera to different preparations of purified VP70 were used (Walsh et al., 1985). One of these (14-07) neutralized virus and inhibited fusion, while the other (14-06) neutralized virus but did not inhibit fusion.

Solid-phase immunoassay. The binding characteristics and the competitive interaction among the monoclonal and polyclonal antibodies were performed by a solid-phase enzyme-linked immunoassay. Fifty ng of purified fusion protein in 100 \(\mu\)l of coating buffer (0.1 M-NaHCO\(_3\), pH 9.9) was adsorbed (4 °C, 18 h) onto polystyrene plates (Dynatech Immunulon-2). The wells were emptied and blocked with 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 1 h at room temperature. For the binding assay, 100 \(\mu\)l of various dilutions of ascitic fluids in 1% BSA–PBS containing 0.1% Tween-20 (PBS–BSA–Tw) was incubated in antigen-coated wells in duplicate for 2 h at 37 °C. After washing with PBS–BSA–Tw, alkaline phosphatase-labelled rabbit anti-mouse IgG was added for 1 h at 37 °C, followed by 200 \(\mu\)l of substrate for 30 min. The absorbance at 410 nm was recorded with a Dynatech MR600 Microelisa reader. Competition among the monoclonal and polyclonal antibodies was performed by simultaneously incubating alkaline phosphatase-labelled MAb with dilutions of unlabelled competitor antibody. All polyclonal and monoclonal antibodies were conjugated to alkaline phosphatase according to the method of Voller et al. (1976).

Neutralization and inhibition of fusion assays. Neutralization ability was determined by a 50% plaque reduction assay; all antibodies with a titre greater than 1:100 in the absence of complement were considered neutralizing. The ability of an antibody to inhibit fusion of previously infected cells was performed as previously described (Walsh & Hruska, 1983). Briefly, monolayers of 2 \(\times\) 10\(^5\) HEp-2 cells were infected with RSV at an m.o.i. of 0.01. Eight h later the medium was removed and the monolayer covered with 1 ml MEM containing the MAb. After 2 days, cell–cell spread of virus was assayed by direct immunofluorescence using a MAb to the nucleocapsid protein. All antibodies were screened initially at 1:100 dilutions, and fusion-inhibiting antibodies were titrated to the highest dilution that gave complete absence of syncytia after 4 days as determined by light microscopy.

PAGE and Western blotting. Five \(\mu\)g of purified VP70 in 500 \(\mu\)l of sample buffer with or without 2-mercaptoethanol (2-ME) was electrophoresed on a 12% SDS–polyacrylamide gel as previously described (Walsh et al., 1985). The sample was either boiled for 3 min or kept at room temperature prior to electrophoresis. Previous work demonstrated that VP70 migrates as a dimer when electrophoresed without prior heat treatment, as a monomer when heated without 2-ME reduction, and as 48K and 23K fragments when treated with 2-ME and heat (Walsh et al., 1985). Following SDS–PAGE, the proteins were transferred to nitrocellulose paper which was cut into strips and incubated for 1 h with 2 ml of buffer (0.05 M-Tris–HCl, 0.9 mM-NaCl pH 7.4, 0.1% Tween) containing 10 \(\mu\)g of MAb. After washing in buffer, the strips were incubated in 2 ml of a 1 : 200 dilution of rabbit anti-mouse IgG antibody (Cappel Laboratories) followed by 200000 c.p.m. \(^{125}\)I-labelled staphylococcal Protein A (IPA, New England Nuclear). The strips were autoradiographed using a Cronex intensifier screen.

RESULTS

Biological activities of MAbs to VP70

The biological activities of the MAbs are listed in Table 1. Only two of the 13 (A8, A9) did not neutralize virus. Five MAbs (L4, A4, A5, A6, R235) neutralized virus and inhibited fusion of
RSV fusion protein epitope map

Fig. 1. Binding characteristics of monoclonal antibodies to VP70 of RSV. Increasing amounts of MAb were incubated in wells coated with 50 ng of purified VP70. Bound antibody was detected by alkaline phosphatase-labelled rabbit anti-mouse IgG. All experiments were performed in duplicate and repeated three times. A representative experiment is shown. (a) Binding characteristics of non-fusion-inhibiting neutralizing MAbs R13-1 (○), R392 (△), R332-4 (□), R136 (▲), R661 (●) and R294 (■). (b) Binding characteristics of fusion-inhibiting neutralizing MAbs A5 (■), L4 (▲), A4 (□), A6 (●) and R235 (○). (c) Binding characteristics of non-neutralizing MAbs A8 (●) and A9 (■).

Table 1. Characteristics of monoclonal antibodies

<table>
<thead>
<tr>
<th>Monoclonal antibody and isotype</th>
<th>Neutralizing activity*</th>
<th>Inhibition of fusion activity†</th>
</tr>
</thead>
<tbody>
<tr>
<td>L4 (G2a), A4 (G1), A5 (G1), A6 (G1), R235 (G2a)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>R661 (G3), R392 (G2a), R294 (G3), R13-1 (G1), 332-4 (G2b), R136 (G2b)</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>A8 (G1), A9 (G1)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>14-06‡</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>14-07‡</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

* Neutralization titre > 1:100 by 50% plaque reduction without complement.
† Inhibits fusion of previously infected cells at titre > 1:100.
‡ Rabbit monospecific polyclonal antibody produced by immunization with purified fusion protein (Walsh et al., 1985).

previously infected cells. Each of these antibodies inhibited fusion at an antibody concentration of 6 to 10 μg/ml. Below this concentration syncytia began to form. The remaining six MAbs neutralized virus but did not inhibit fusion.

Binding of MAbs to VP70 and competition binding

The binding characteristics of the monoclonal antibodies described a spectrum of curves (Fig. 1a, b, c). Although the binding characteristics may be affected by the avidity of the alkaline phosphatase-labelled rabbit anti-mouse IgG antibody, no apparent relationship between avidity and isotype was noted. The fusion-inhibiting MAbs all demonstrated relatively high avidity (i.e. plateau $A_{410}$) whereas the neutralizing MAbs bound with high and low avidity (Fig. 1). Thus, although neutralization was not related to binding characteristics, the ability to inhibit fusion was correlated with relatively high avidity binding to VP70. Both non-neutralizing MAbs bound to VP70 with low avidity (Fig. 1c).
Table 2. Epitope assignments of anti-VP70 monoclonal antibodies

<table>
<thead>
<tr>
<th>Labelled MAb</th>
<th>R661, R392, R294</th>
<th>R136, R332-4, R13-1†</th>
<th>L4‡</th>
<th>A4, A5, A6‡</th>
<th>R235‡</th>
<th>A8, A9§</th>
</tr>
</thead>
<tbody>
<tr>
<td>R661, R392, R294†</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>±</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>L4‡</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>±</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>A4, A5, A6‡</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>±</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>A8, A9§</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* Degree of competition: +, complete competition (>90%); ±, partial competition (50 to 75%); -, minimal competition (<45%).
† Neutralizing, non-fusion-inhibiting MAbs.
‡ Neutralizing, fusion-inhibiting MAbs.
§ Non-neutralizing, non-fusion-inhibiting MAbs.

To examine the topographical characteristics of VP70, the epitopes that subserved neutralization and fusion inhibition were defined by competitive binding. Four of the fusion-inhibiting MAbs (L4, A4, A5, A6), three neutralizing MAbs (R661, R294, R392) and the two non-neutralizing MAbs (A8, A9) were labelled with alkaline phosphatase. Competition was arbitrarily defined as complete, partial or minimal if the unlabelled antibody prevented attachment of labelled antibody by >90%, 50 to 75% or <45% respectively when the competitor was used at 10 μg/ml. Competition by two 17D yellow fever virus monoclonal antibodies served as a control. The competitor concentration was at least 100 times greater than the plateau concentration of the binding curves (Fig. 1). Complete competition (>90%) was required for classification as a distinct epitope. The competition criteria were partially based upon the values obtained, and are similar to criteria used by other investigators (Stone & Nowinski, 1980; Iorio & Bratt, 1983). Although Stone & Nowinski suggest that partial competition between antibodies may result from differences in avidity, we did not find that this occurred. Both low (R294, R661) and high (R13-1, R392) avidity antibodies gave comparable competition with a labelled high avidity antibody (R392). The results of these experiments are summarized in Table 2.

At least two and perhaps three fusion-inhibiting epitopes on VP70 were described by competitive binding. One epitope was described by L4, and a second fusion-inhibiting epitope by A4, A5 and A6. A third possible epitope was described by R235, as it failed to compete partially with labelled A4, A5 or A6 and only partially competed with labelled L4. R235, if used at high concentrations, may have given greater competition with labelled L4, as suggested by Fig. 2(a). Unfortunately, R235 did not retain specific binding activity when labelled with alkaline phosphatase and thus we could not determine whether unidirectional competition occurred between these fusion-inhibiting MAbs and R235. Fig. 2 graphically illustrates competition curves between MAbs representing the defined epitopes. Fig. 2(a) illustrates that complete competition with labelled L4 occurs only with the homologous unlabelled antibody, while partial competition by R235 and A6 was noted. A4 and A5 also gave partial competition with labelled L4 (data not shown). This suggests partial overlap of these fusion-inhibiting epitopes. There was minimal competition between labelled L4 and the neutralizing and non-neutralizing MAbs. Fig. 2(c) illustrates complete competition between unlabelled A6 and labelled A4 and minimal competition by L4 and R235. A4, A5 and A6 all demonstrated complete cross-competition as outlined in Table 2. The neutralizing (R392, R661) and non-neutralizing (A8) MAbs also showed minimal competition with labelled A4.

A single epitope was described by the six non-fusion-inhibiting neutralizing MAbs (Table 2). Partial competition occurred between labelled R661 and A6 (Fig. 2d) and A4 and A5 (not shown) but minimally with R235 or L4, suggesting a possible spatial proximity of the neutralizing epitope with the fusion-inhibiting epitopes.
The two non-neutralizing antibodies cross-competed but did not compete with other MAbs, and thus may describe a single non-neutralizing epitope (Fig. 2b). In all, four and possibly five distinct epitopes on the fusion protein were described by the 13 MAbs in competitive binding studies.

In similar competition experiments, both polyclonal rabbit sera to VP70 demonstrated complete competition with the labelled neutralizing and fusion-inhibiting MAbs. Since one of these competing polyclonal sera (14-06) did not inhibit fusion, we examined its ability to block the in vitro activity of fusion-inhibiting MAbs. Monolayers of HEp-2 cells in 24-well plates were
Fig. 3. PAGE and Western blotting of VP70. Five μg of purified VP70 was electrophoresed under various conditions on a 12% SDS-polyacrylamide gel, transferred to nitrocellulose paper, and probed with MAbs. (a) VP70 with no pretreatment. All MAbs except R235 (lane 7) reacted with the dimer of VP70. A rabbit anti-VP70 serum (14-07) also reacted with the dimer (lane 9). Standard mol. wt. markers are shown in lane 1. (b) VP70 with heat pretreatment. All MAbs, including R235 (lane 6), reacted with the monomer of VP70. (c) VP70 with 2-ME and heat pretreatment. Only two MAbs, L4 (lane 1) and A8 (lane 2), reacted with the 48K fragment of VP70. A9 also reacted with the 48K fragment (not shown). Rabbit serum (14-07) reacted with both the 48K and 23K fragments (lane 6).
infected with RSV at an m.o.i. of 0.01 and incubated for 8 h. The medium was removed and overlaid with medium containing MAb alone, rabbit serum (14-06) alone or MAb plus rabbit serum. The MAb was used at the lowest concentration which inhibited fusion (6 to 10 μg/ml) and the polyclonal rabbit serum at a 1:100 dilution. After 3 days the cell sheet was examined for syncytium formation. As described previously, the rabbit serum alone did not inhibit fusion while the MABs prevented syncytium formation. The cells incubated with the mixture of MAB and polyclonal serum did not develop syncytia. Thus, although the polyclonal serum competed with the MABs in a solid-phase assay, it did not block their biological activity.

To investigate further the binding characteristics of the MABs to VP70, Western blot analysis was performed using purified VP70 as the antigen. This demonstrated that all MABs except the fusion-inhibiting antibody R235 reacted with the dimer of VP70 (Fig. 3a) while all MABs including R235 reacted with the monomer of VP70 (Fig. 3b). This finding is consistent with the competitive binding studies which suggest that R235 describes a fusion-inhibiting epitope distinct from the two epitopes described by L4 and A4, A5 and A6.

When VP70 was treated with heat and 2-ME prior to electrophoresis, L4, A8 and A9 reacted strongly with the 48K fragment (Fig. 3c), but none of the other MABs reacted with either fragment. A weak reactivity to the 23K fragment was noted with L4, suggesting some degree of cross-reactivity between these fragments.

**DISCUSSION**

Recent studies have identified the role of the RSV fusion protein in viral infectivity and spread, and animal experiments suggest that antibody to this protein may be important in immunity (Taylor *et al.*, 1984; Walsh *et al.*, 1984b). In this report, we describe the topographical relationship of important epitopes on the fusion protein. Recently, MABs have been used to evaluate clinical RSV isolates for possible strain variation (Anderson *et al.*, 1985). The clinical importance of any strain differences, however, is unclear. A better appreciation of significant strain variation might be obtained by using monoclonal antibodies to the biologically important epitopes of the fusion protein as defined in this report. A similar approach has been used to evaluate strain variation of Newcastle disease virus (Iorio *et al.*, 1984).

Four or five antibody-binding sites, or epitopes, on the RSV fusion protein are described based upon biological activity, solid-phase competitive binding and Western blotting assays using 13 MABs. The partial competition between MABs directed to these epitopes may reflect topographical proximity of the binding sites, although changes in the tertiary structure of the antigen induced by the binding of one antibody may alter the binding of a second antibody to a distinct epitope. Thus, the distance between epitopes or their actual spatial arrangement cannot be accurately determined from these studies. Binding of antibody to two or three of these epitopes inhibits the fusion function of VP70 and neutralizes virus while a separate epitope subserves neutralization only. Although not conclusive, the Western blot data do suggest that one of the fusion-inhibiting neutralizing MABs, R235, binds to a site different from the other four, L4 and A4, A5 and A6. It is of interest that the fusion protein possesses distinct epitopes which subserve fusion and neutralization. Presumably, antibody that binds to one of the fusion epitopes neutralizes virus by preventing fusion of viral and cellular membranes, thus blocking viral penetration. Antibody binding to the neutralizing epitope probably neutralizes virus by a different mechanism, perhaps by steric hindrance of virus attachment, or by prevention of uncoating or transcription of the viral genome.

Although as many as three fusion-inhibiting epitopes were found, it is not known whether any of the antibodies actually bind to that sequence of the fusion protein which mediates fusion of cellular membranes. The active fusion site of the Sendai virus fusion protein and of the influenza haemagglutinin are unique short peptide regions (Atassi & Webster, 1983; Choppin & Scheid, 1980). Recently, the amino acid sequence of the RSV fusion protein, and the presumed active fusion region, have been reported (Collins *et al.*, 1984). Alternatively, antibody may sterically inhibit the active fusion site from its postulated interaction with cellular membranes (Hsu *et al.*, 1981).
The study of paramyxovirus infectivity prompted Merz et al. (1981) to suggest that a successful vaccine should induce antibody that inhibits the function of the fusion protein. Although fusion-inhibiting antibody can be induced by immunization of animals with purified VP70, antibody which neutralizes virus but does not inhibit fusion may also result (Walsh et al., 1985). A more uniform and reliable immune response to vaccination is desirable, especially in light of the problems following vaccination with a formalin-inactivated RSV vaccine (Kim et al., 1969). Newer strategies for the development of viral vaccines include the use of short peptide fragments synthesized from the known amino acid sequence of viral proteins (Arnon et al., 1983). Immunization with a fragment of the RSV fusion protein which consistently induces fusion-inhibiting and neutralizing antibody is a reasonable approach to vaccine development. The binding sites that subserve fusion inhibition are candidates for such a synthetic vaccine. The ability of L4, a fusion-inhibiting neutralizing MAb, to bind to the 48K fragment of reduced, denatured VP70 suggests that this fusion-inhibiting epitope may be amenable to the synthetic peptide approach to vaccine development since the antigenic site remains reactive under denaturing conditions and may be a continuous epitope region. Analysis of enzymic or cyanogen bromide cleavage fragments of VP70 will be useful to localize this epitope more precisely.

A theoretical problem when peptide fragments are used for immunization is the presence of blocking antibodies which may prevent the peptide fragment-induced antibodies from binding to the native protein in vivo. Blocking of neutralization by a non-neutralizing monoclonal antibody that competed with a neutralizing MAb in a competitive binding assay has been described (Massey & Schochetman, 1981). We did not observe blocking of the in vitro activity of any fusion-inhibiting MAb by a non-fusion-inhibiting polyclonal antiserum despite competition in the solid-phase competitive binding assay. The reasons for this apparent discrepancy can only be speculative, but similar observations with neutralization by MAbs have been reported (Schlesinger et al., 1984).

The authors wish to thank Margaret English for technical assistance and Joanne Prives for preparation of the manuscript. This work was supported in part by a research grant from Praxis Biologics, Rochester, New York, and in part by Contract N01-All-22665 between Georgetown University and the National Institute of Allergy and Infectious Diseases.

REFERENCES


RSV fusion protein epitope map


(Received 9 July 1985)